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Exercise training increases CISD family protein expression in murine skeletal muscle and white adipose tissue

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ABSTRACT

Mitochondrial function in skeletal muscle and white adipose tissue (WAT) declines with aging and the progression of type 2 diabetes and insulin resistance. Although exercise increases mitochondrial biogenesis and function in both tissues, the molecular mechanisms are not fully understood. CDGSH iron sulfur domain-containing proteins (CISDs) are a novel family of proteins that regulate mitochondrial activity and biogenesis. However, the relationship between exercise and CISD expression is unclear. We addressed this in the present study by examining changes in the expression of CISDs and mitochondrial proteins in skeletal muscle and WAT of mice subjected to chronic exercise training. Mice were randomly assigned to either the sedentary or exercise group and were housed for 4 weeks in a standard cage without or with a running wheel, respectively. CISD and mitochondrial protein levels in the plantaris and soleus muscles and epididymal WAT were evaluated by western blotting. Chronic exercise increased CISD1 and CISD2 as well as mitochondrial protein expression in plantaris muscle and WAT but not soleus muscle. Moreover, this exercise-induced adaptation was strongly correlated with mitochondrial protein expression. Thus, mitochondrial biogenesis induced by chronic exercise coincides with the expression of CISDs in specific tissues, which may be critical for the maintenance of mitochondrial integrity.

1. Introduction

A decline in mitochondrial function in skeletal muscle and white adipose tissue (WAT) is associated with aging and the progression of type 2 diabetes (T2D) and insulin resistance [1–4]. Accordingly, mitochondrial dysfunction is observed in the skeletal muscle of patients with type 2 diabetes and elderly people [5,6]. In WAT of obese human, mitochondrial gene expressions were also lower than these of lean human [7]. Interestingly, overexpression of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α, a master regulator of mitochondrial biogenesis, inhibited protein degradation and suppressed induction of ubiquitin ligases in skeletal muscle with disuse atrophy [8]. In addition, adipose tissue-specific PGC-1α knockout mice showed reduced expression of mitochondrial genes and developed insulin resistance when fed a high-fat diet [9]. Thus, interventions that improve mitochondrial function and enhance mitochondrial biogenesis in skeletal muscle and WAT can potentially slow aging and prevent T2D and insulin resistance.

Exercise is known to enhance mitochondrial biogenesis and function in skeletal muscle and WAT [10]. It also improves insulin sensitivity in healthy and insulin-resistant individuals and inhibits the progression of T2D [1]. Aerobic training increased mitochondrial content and insulin sensitivity in T2D patients, and these improvements were correlated [11]. Additionally, exercise training during aging was shown to increase mitochondrial biomass in skeletal muscle [12]. However, the molecular mechanism of exercise-induced mitochondrial biogenesis is not fully understood.

PGC-1α is the most widely studied regulator of exercise-induced mitochondrial changes in skeletal muscle and WAT. Several studies have reported that exercise increases PGC-1α levels in skeletal muscle [13,14] and WAT [15,16]. However, exercise-induced mitochondrial biogenesis in muscle was not diminished in muscle-specific PGC-1α knockout mice [17,18], suggesting that other molecules are involved in this process.

CDGSH iron sulfur domain-containing proteins (CISDs) regulate mitochondrial activity and biogenesis. CISD1 (also known as

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mitoNEET and ZCD1) is a target of pioglitazone and other thiazolidinediones [19]. TT01001, an orally administered small molecule drug designed on based on the pioglitazone structure was shown to bind to CISD1 and improve mitochondrial function and glucose tolerance in diabetic db/db mice [20], suggesting that CISD1 may at least partly mediate the anti-diabetic effects of piogitazone. Adipose tissue-specific CISD1 overexpression increased mitochondrial content and insulin sensitivity [21], and in cultured cells, it also increased mitochondrial protein expression [22]. CISD2 (also known as ZCD2, Noxp70, NAF-1, and Miner 1) regulates mitochondrial biogenesis and activity; CISD2 protein levels in skeletal muscle were found to decline with aging and its overexpression in mice abrogated aging-induced mitochondrial dysfunction and muscle atrophy [23]. CISD2 knockout mice showed decreased expression of mitochondrial biogenesis-related genes relative to their wild-type counterparts as well as abnormal mitochondrial morphology in WAT [24].

Based on the above evidence, we speculated that chronic exercise increases CISD expression in skeletal muscle and WAT, thereby stimulating mitochondrial biogenesis. To test this hypothesis, we compared the expression of CISD1 and CISD2 in skeletal muscle and WAT in mice subjected to exercise regimens for 4 weeks and those that remained sedentary during this period.

2. Methods

2.1. Animals

All experimental procedures and animal care were approved by the Committee on Animal Care at Ritsumeikan University. Male C57BL/6J mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). Mice were housed under controlled conditions on a 12:12-h light/dark cycle (with lights on from 8:00–20:00), with free access to food and water.

2.2. Chronic exercise intervention

After 1 week of acclimatization, 12-week-old mice were randomly assigned to either the sedentary or exercise group ($n = 6$ per group). Sample size was determined from pilot experiments and a previous study [25]. Each group of mice was housed in a standard cage ($n = 3$/cage) without (sedentary group) or with (exercise group) a running wheel for 4 weeks. Body weight, running distance, and food intake was measured each week. The morning (8:00–11:00) after the last day of exercise, mice were euthanized by cervical dislocation, and the plantaris and soleus muscles and epididymal WAT were removed, frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.3. Single-day exercise intervention

To distinguish between chronic and acute effects of exercise, mice were subjected to single-day exercise intervention. After initial acclimatization, 8-week-old mice were randomly assigned to the sedentary ($n = 5$) or exercise ($n = 6$) group; the sample size was determined from pilot experiments and a previous study [26]. Each group of mice was individually housed in a standard cage without (sedentary group) or with (exercise group) a running wheel for 24 h. The next morning (8:00–11:00), body weight, running distance, and food intake were measured, and the mice were sacrificed by cervical dislocation. The plantaris and soleus muscles and epididymal WAT were rapidly excised, frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.4. Western blotting

Western blotting was performed as previously described [27]. The plantaris and soleus muscle tissues were lysed and homogenized in 10–15 vol of ice-cold radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholte, 1 mM ethylenediamineteraacetic acid, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with cOmplete mini protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysates were incubated for 60 min at 4 °C, and then centrifuged at 14,000 × *g* for 20 min at 4 °C. The supernatant was collected for protein assay. Epididymal WAT was lysed and homogenized in two volumes of ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors. The lysates were incubated for 60 min at 4 °C, and then centrifuged at 800 × *g* for 1 min at 4 °C. The fat cake was removed, and the infranatant was centrifuged at 14,000 × *g* for 20 min at 4 °C. After another round of centrifugation for 20 min, the infranatant was collected and protein concentration was determined with the Protein Assay Bicinchoninate Kit (Nacalai Tesque, Kyoto, Japan). Lysates of equal volume were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using a Mini-slab size electrophoresis apparatus (ATTO Corp., Tokyo, Japan). The separated proteins were transferred to a 0.45-μm pore polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) at 100 V for 30 min in Towbin buffer (25 mM Tris, 192 mM glycine, and 15% or 20% methanol) using a Criterion blotter (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat dry milk (NFDM)/Tris-buffered saline with 0.01% Tween-20 (TBST) for 30 min at 25 °C, the membrane was washed twice for 3 min each in TBST, and then incubated overnight at 4 °C in 5% bovine serum albumin in TBST with primary antibodies against CISD1 (1:5000; Cell Signaling Technology, Danvers, MA; #83775) and CISD2 (1:5000; Abcam, Cambridge, UK; ab167109), as well as Total OXPHOS Rodent WB Antibody Cocktail (1:3000; Abcam; ab110413). The membrane was washed twice for 5 min each in TBST and then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000; Cell Signaling Technology; #7074 or #7076) or VeriBlot for IP Detection Reagent (1:500; Abcam; ab131366) in 1% NFDM/TBST for 60 min at 25 °C. After washing three times in TBST for 5 min each, chemiluminescence quantification was performed using the Laminata Forte Western HRP Substrate (Millipore), followed by image acquisition on an ImageQuant LAS 4000 system (GE Healthcare, Little Chalfont, UK).

2.5. Coomassie brilliant blue (CBB) staining

Equal protein loading in SDS-PAGE was confirmed by CBB staining. The membrane was briefly rinsed in distilled water and then stained in CBB solution (0.25% CBB R-250, 40% methanol, and 10% acetic acid) for 10 min at 25 °C. After rinsing three times in distilled water, the stained membrane was washed three times for 15 min each in destaining solution (40% methanol and 10% acetic acid) followed by three rinses in distilled water. The membrane was dried, and digital images were acquired with the ImageQuant LAS 4000 system.

2.6. Image analysis and quantification

Images of the immunoblot and CBB-stained membrane were analyzed using the Fiji image processing package based on ImageJ software (National Institutes of Health, Bethesda, MD, USA) [28]. The signal intensity of protein bands in the immunoblot was normalized to CBB staining intensity and converted to a value relative to the sedentary control group.

2.7. Statistical analysis

Statistical analyses were performed using open source R software [29]. Regardless of whether the compared groups showed homo- or

heteroscedasticity, data for the sedentary and exercise groups were compared with the two-tailed Welch's *t*-test. The relationship between mitochondrial protein and CISD expression was assessed with Pearson's product-moment correlation coefficient. All bar and scatter plots were generated with the ggplot package of R software. The statistical significance level was set at $P < 0.05$. Results are presented as dot plots and the mean ± standard error.

3. Results

3.1. Effect of 4-week exercise training on CISD expression

3.1.1. Physiological characteristics of exercise-trained and sedentary mice

Exercise training for 4 weeks did not significantly alter body weight. No significant difference in the weight of the plantaris muscle was found between the two groups. On the other hand, the weight of the soleus muscle in exercised mice tends to be higher than that in sedentary mice. Additionally, exercise training significantly decreased the weight of epididymal WAT (data not shown). These results are consistent with previous reports [25,30].

3.1.2. CISD and mitochondrial protein expression in skeletal muscle

Exercise training for 4 weeks significantly increased CISD1 and CISD2 protein levels in the plantaris muscle (Fig. 1A). The levels of mitochondrial oxidative phosphorylation proteins including NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit (NDUFB)8, succinate dehydrogenase [ubiquinone] iron-sulfur subunit (SDHB), cytochrome b-c1 complex subunit (UQCRC)2, ATP synthase subunit alpha (ATP5A), and mitochondrial cytochrome c oxidase (MTCO)1

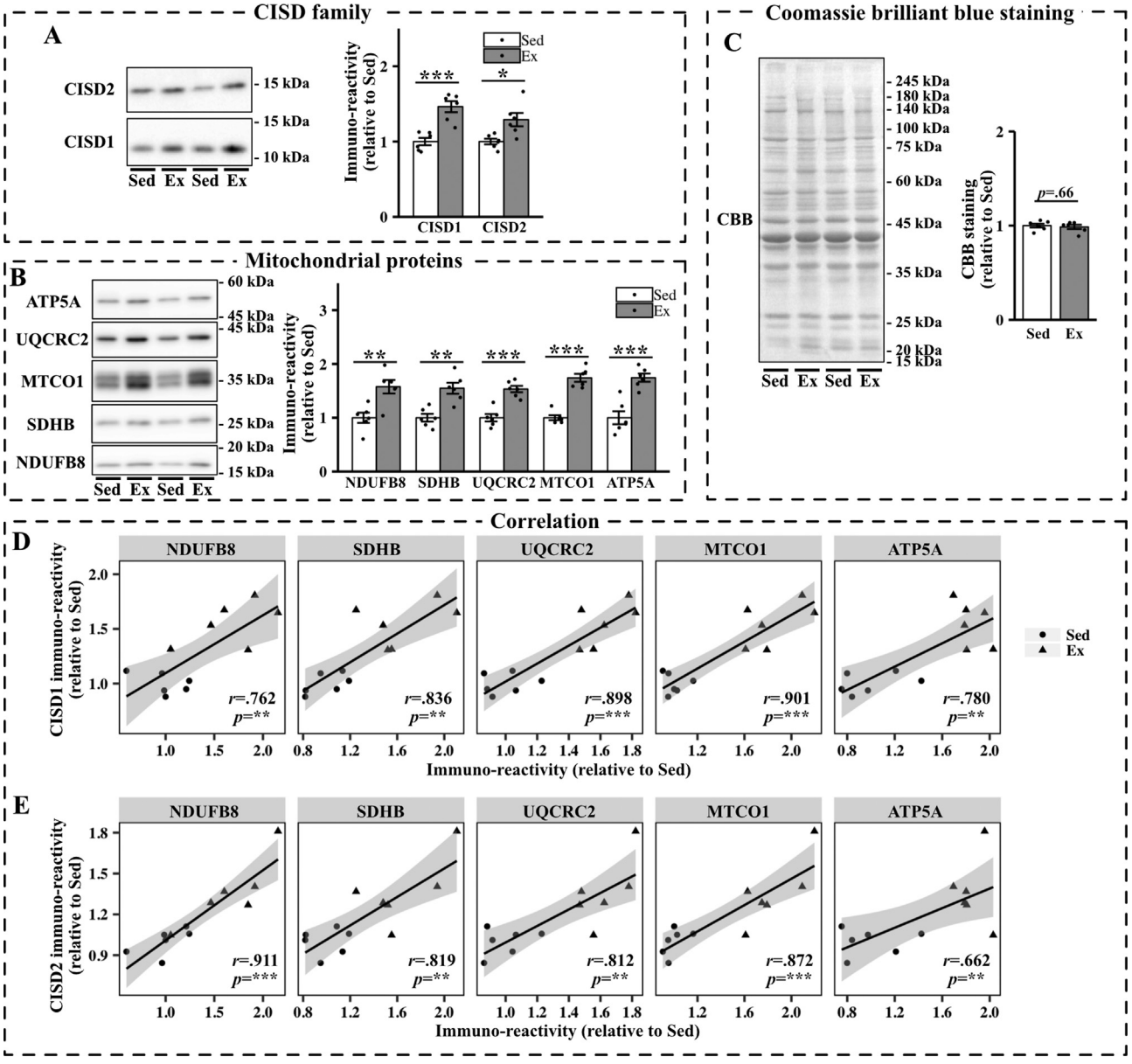

Fig. 1. Effect of 4 weeks of exercise on CISD and mitochondrial protein expression in the plantaris muscle. (A–C) Representative images and quantitative analysis of CISD (A) and oxidative phosphorylation protein (B) levels and CBB staining (C) in the plantaris muscle of sedentary and exercise groups. (D, E) Scatter plots showing the levels of CISD1 (D) and CISD2 (E) vs. those of mitochondrial proteins. Shaded areas show 95% confidence limits of the fitted line. *P < 0.05, **P < 0.01, ***P < 0.001 vs. sedentary group. Sed: sedantary control; Ex: exercise group.

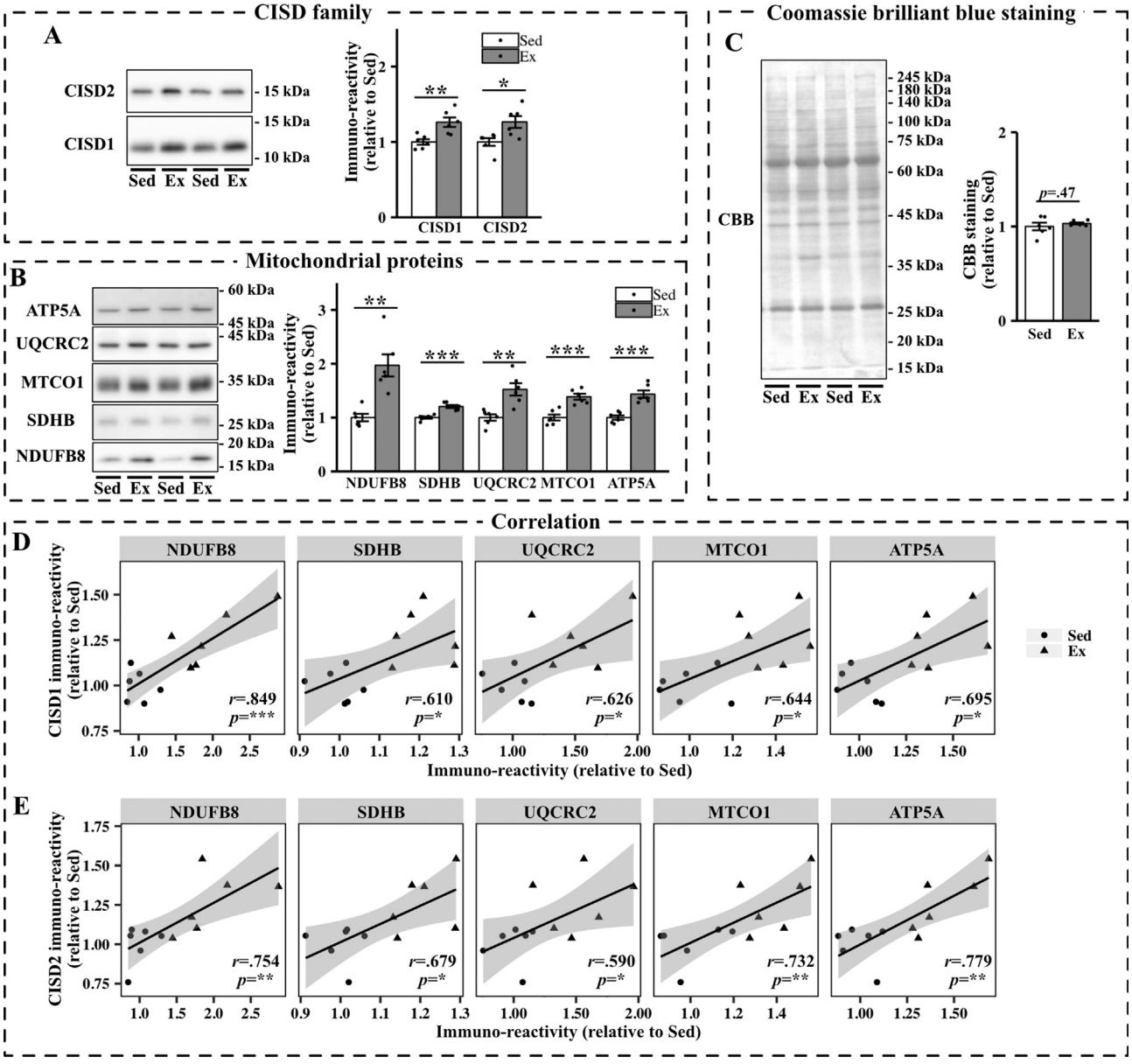

Fig. 2. Effect of 4 weeks of exercise on CISD and mitochondrial protein expression in epididymal WAT. (A–C) Representative immunoblots and quantitative analysis of CISD (A) and oxidative phosphorylation protein (B) levels and CBB staining (C) in the epididymal WAT of sedentary and exercise groups. (D, E) Scatter plots showing the levels of CISD1 (D) and CISD2 (E) vs. those of mitochondrial proteins. Shaded areas show 95% confidence limits of the fitted line. *P < 0.05, **P < 0.01, ***P < 0.001 vs. sedentary group. Sed: sedantary control; Ex: exercise group.

were also significantly increased in the exercise group (Fig. 1B). We examined the association between CISD and mitochondrial protein expression in the plantaris muscle and found that they were positively correlated (Fig. 1D and E). On the other hand, we did not detect exercise-induced increases in CISDs and mitochondrial proteins in the soleus muscle, which showed decreases in the protein contents of CISD2 and SDHB (data not shown). There was no difference in CBB staining between the two groups in the plantaris (Fig. 1C) or the soleus muscle (data not shown).

3.1.3. CISD and mitochondrial protein expression in WAT

Four weeks of exercise training significantly increased CISD1 and CISD2 protein levels in epididymal WAT (Fig. 2A), while NDUFB8, SDHB, UQCRC2, ATP5A, and MTCO1 expression was also upregulated (Fig. 2B). CISD and mitochondrial protein levels were positively correlated in epididymal WAT (Fig. 2D and E). There was no significant difference in CBB staining between the two groups (Fig. 2C).

3.2. Effect of single-day exercise on CISD expression

3.2.1. Physiological characteristics of exercise-trained and sedentary mice

No significant differences in body, muscle, or epididymal WAT weight were observed between mice that were sedentary and those that were exercised for 1 day (data not shown).

3.2.2. CISD and mitochondrial protein expression in skeletal muscle

One day of exercise did not significantly alter CISD (Fig. 3A) or mitochondrial (Fig. 3B) protein expression in plantaris muscle.

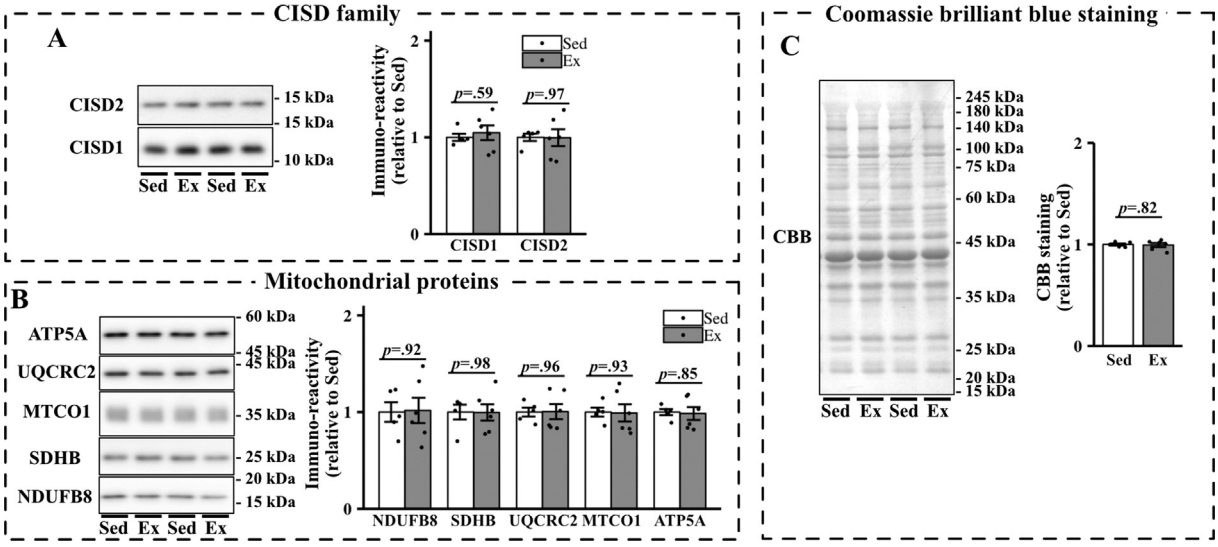

Fig. 3. Effect of a single day of exercise on CISD and mitochondrial protein expression in the plantaris muscle. (A–C) Representative images and quantitative analysis of CISD (A) and oxidative phosphorylation protein (B) levels and CBB staining (C) in the plantaris muscle of sedentary and exercised groups.

There was no statistically significant difference in CBB staining between the two groups (Fig. 3C).

3.2.3. *CISD and mitochondrial protein expression in WAT*

We did not detect any changes in the expression of CISD (Fig. 4A) or mitochondrial (Fig. 4B) proteins in epididymal WAT. There was no difference in CBB staining between the two groups (Fig. 4C).

4. Discussion

The results of this study demonstrate that 4 weeks of exercise increased the expression of CISDs as well as mitochondrial proteins in plantaris muscle and WAT but not in soleus muscle. In contrast, a single day of exercise did not alter the expression of these proteins in the plantaris muscle and WAT. This is the first report showing that CISD protein expression is upregulated in these tissues by exercise training.

Previous studies have demonstrated that CISD1 and CISD2 regulate mitochondrial biogenesis and function in skeletal muscle and WAT using transgenic and knockout mice [21,23,24,31]. However, it was not clear whether CISD expression was related to mitochondrial biogenesis under physiological conditions. Although PGC-1β and PGC-1-related coactivator 1 were identified as mitochondrial regulatory proteins [32,33], exercise intervention did not alter their expression in skeletal muscle and WAT [15,34], suggesting that the upregulation of the two proteins does not mediate exercise-induced mitochondrial adaptation. In order to clarify the molecular mechanism by which exercise enhances mitochondrial biogenesis and function in skeletal muscle and WAT, we examined the response to exercise of other proteins that are known to regulate mitochondrial biogenesis—namely, CISDs. We found that chronic exercise increased the expression levels of CISDs, as well as mitochondrial proteins, in plantaris muscle and epididymal WAT. Additionally, their expression was significantly correlated with that of mitochondrial proteins in both plantaris muscle and WAT. However, exercise training did not alter CISD and mitochondrial

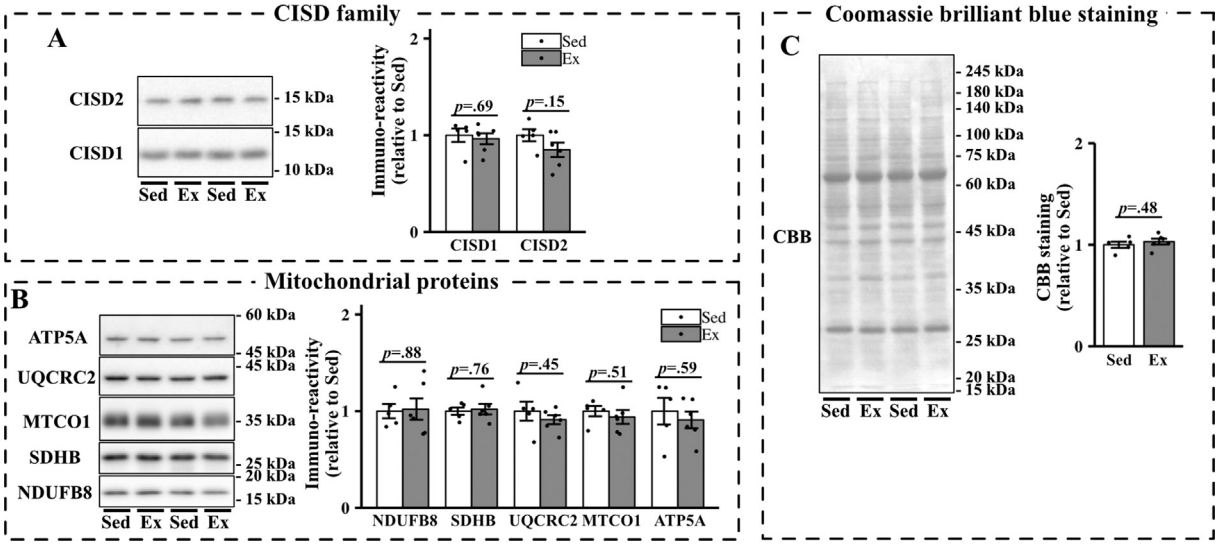

Fig. 4. Effect of a single day of exercise on CISD and mitochondrial protein expression in epididymal WAT. (A–C) Representative images and quantitative analysis of CISD (A) and oxidative phosphorylation protein (B) levels and CBB staining (C) in epididymal WAT of sedentary and exercised groups. Sed: sedantary control; Ex: exercise group.

protein levels in soleus muscle, which lacks wheel running-induced mitochondrial adaptivity [17,35].

To distinguish between chronic and acute effects of exercise, mice were subjected to exercise intervention for a single day. Some mitochondrial proteins adapt quickly to physiological stimuli [36], and CISD1 has been reported to be rapidly induced by translational machinery [37]. For this reason, exercise cessation 24–48 h before sampling, which is often used to avoid the effect of acute exercise [25] [38], might downregulate enhanced mitochondrial proteins and mask exercise-induced adaptation. Therefore, mice of the chronic exercise group were immediately sacrificed after the 4-week exercise period in order to minimize the effect of exercise cessation, but this study design does not rule out the possibility that increases in the expression of target proteins were induced by the final bout of exercise rather than by chronic effects. However, since single-day exercise intervention did not alter the expression levels of CISDs and mitochondrial proteins in plantaris muscle and epididymal WAT, we conclude that the changes observed in mice that were exercised for 4 weeks were the result of chronic adaptation and not due to an acute response.

One limitation of this study is that it did not definitively establish a causal relationship between the expression of CISDs and that of mitochondrial proteins; as such, it is unclear whether CISDs directly mediate exercise-induced mitochondrial biogenesis. To clarify this point, it is first choice to investigate whether knockout mice of candidate genes suppress or diminish exercise-induced mitochondrial biogenesis [16]. However, previous studies have reported that exercise-induced mitochondrial biogenesis was not diminished in PGC-1α knockout mice [17,18]. Although this suggests that PGC-1α does not mediate exercise-induced mitochondrial biogenesis, it is possible that complete genetic ablation of the gene can lead to adaptations that mask the physiological role of PGC-1α. In fact, modest overexpression of PGC-1α by electroporation resulted in adaptations similar to those induced by exercise such as increased mitochondrial biogenesis, insulin sensitivity, and palmitate oxidation, which is partly inconsistent with the phenotype of PGC-1α transgenic mice [39]. Therefore, additional studies using different physiological models and loss- and gain-of-function approaches are needed to elucidate the physiological roles of CISDs.

In conclusion, the results of this study demonstrate that exercise training for 4 weeks increased the expression of CISD1 and CISD2 as well as mitochondrial proteins in plantaris muscle and epididymal WAT. These adaptations were not detected in soleus muscle. Thus, the expression of CISDs in plantaris muscle and epididymal WAT induced by chronic exercise may be critical for the maintenance of mitochondrial integrity.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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